CHEMICAL ENGINEERING TRANSACTIONS
VOL. 43, 2015
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Chief Editors: Sauro Pierucci, Jiří J. Klemeš

**ISBN** 978-88-95608-34-1; **ISSN** 2283-9216
DOI: 10.3303/CET1543107

# Improved *Saccharomyces Cerevisiae* Growth on Cheese Whey by Controlling Enzymatic Lactose Hydrolysis

Isabella Pisano*,[ab], Gennaro Agrimi[ab], Guglielmo Grosso[a], Maria Concetta Mena[a], Maria Antonietta Ricci[ab], Luigi Palmieri [abc]

[a]Department of Biosciences, Biotechnologies and Biopharmaceutics, University of Bari "Aldo Moro", Via Edoardo Orabona 4, 70125 Bari, Italy
[b]CIRCC - Interuniversity Consortium Chemical Reactivity and Catalysis, Via Celso Ulpiani 27, 70126 Bari, Italy
[c]CNR Institute of Biomembranes and Bioenergetics (IBBE), via Amendola 165/A, 70126, Bari, Italy
isabella.pisano@uniba.it

Whey generated in cheese manufacturing poses serious environmental issues that limit process profitability. The innovation in the dairy sector recognizes the "bio-refinery" as a key to successful handling of whey disposal and economic rise. Cheese whey valorisation is a complex process involving multiple technologies that might lead to value-added products (biomass, fine or bulk chemicals). This work focuses on the optimization of a fermentation process using whey as growth medium and carbon source. Lactose, which is abundant in whey, is a valuable carbon source. However, the microorganism more widely used in industrial fermentation processes, the yeast *Saccharomyces cerevisiae*, is not a lactose-fermenting yeast. We set up an innovative biotechnological process for the production on large scale of a not-genetically modified yeast biomass that can be used in different contexts, such as bread making, production of probiotics, nutraceuticals, bio-active molecules. In order to use the cheese whey as raw material for the cultivation of *S. cerevisiae* and to overcome the limitations in the use of lactose we used and externally added the enzyme β-galactosidase. The careful optimization of the amount of added enzyme allowed the gradual release by hydrolysis and the simultaneous consumption of glucose and galactose with a consequent decrease of ethanol and an increase of the biomass produced.

## 1. Introduction

A world production over $10^8$ t/year of cheese whey is estimated. It cannot be simply used as animal feed but could represent an attractive raw material for the production of many value-added molecules (Mollea 2013), making whey not only a waste but also a valuable resource.
Whey is a turbid greenish-yellow liquid and constitutes the watery portion after the separation of fat and caseins from whole milk. Costituents of whey are mainly water (92 %), lactose (5 %), whey proteins and fats (1.9 %) and soluble salts (0.9 %). The chemical, physical and microbiological properties are extremely variable, depending on the manufacturing process (fresh or hard cheeses), the milk origin (cow or goat), the season in which milk is produced (González Siso 1996).
Due to its high biological (40,000 ppm) and chemical oxygen demand (60,000 ppm), mainly due to high lactose content, cheese whey disposal requests large amount of industrial capital to avoid serious environmental problem (Ghaly 2007). Thus, there is an obvious need for finding a solution to the whey disposal problem and the "biorefinery", in which a residual biomass, such as whey, is used for the production of different products, such as animal feed, bulk and fine-chemicals, could be the key to success.
The future trend for cheese factories is to move towards zero discharge, i.e., move away from high disposal costs and find more environmentally friendly and profitable applications for lactose. A number of processes have previously been described in which yeasts are used to produce bioethanol (Sansonetti 2010) or active microbial biomasses such as lactic acid starters and baker's yeast (Champagne 1990). This article presents a

Please cite this article as: Pisano I., Agrimi G., Grosso G., Mena M.C., Ricci M.A., Palmieri L., 2015, Improved saccharomyces cerevisiae growth on cheese whey by controlling enzymatic lactose hydrolysis, Chemical Engineering Transactions, 43, 637-642
DOI: 10.3303/CET1543107

alternative strategy to use surplus whey, namely its upgrading to the role of a raw material for cost-efficient production of microbial biomass.
Saccharomyces cerevisiae is one of the oldest products of industrial fermentation and is still one of the most important biotechnological products because of its several industrial applications. Currently the substrates traditionally used for producing yeast biomass are simple sugars, in particular sucrose, and sugar beet molasses or sugar cane. Thanks to its complex chemical composition cheese whey can potentially be used as growth medium and carbon source, it is inexpensive and available locally. Since S. cerevisiae cannot utilize lactose, the main whey carbon source, (Domingues 2010), various approaches have been suggested: conversion of lactose to glucose and galactose by free and immobilized enzymes or by chemical hydrolysis, conversion of lactose to lactic acid, and genetic manipulation in order to enable S. cerevisiae to directly use lactose as a carbon substrate (Champagne 1990, Porro 1992).
This study was aimed at investigating the production of yeast biomass using cheese whey in which lactose is hydrolysed by low amounts of externally added β-galactosidase and glucose and galactose released are simultaneously fermented. Large-scale industrial fermentations are characterized by high cell densities. In order to achieve optimal biomass yields and minimal byproduct formation control of aeration and substrate feeding are fundamental. In the cheese whey fermentation process investigated in this study, the control of carbohydrate utilization is achieved using a low dosage of the enzyme β-galactosidase in order to ensure the simultaneous use of glucose and galactose released and avoid negative regulatory effect of yeast metabolism such as the “Crabtree effect”. This allows to maximize the amount of biomass produced and minimize unwanted alcohol formation.

## 2. Materials and methods

### 2.1 Strain

A strain, named by us PAN, isolated from commercial compressed baker’s yeast (Lievital; Lievitalia S.p.a., Italy) was used. After reactivation of commercial baker’s yeast by a overnight culture in YPD liquid medium, containing 10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose, cells were sampled and plated after serial dilution on YPD solid medium. A single colony was selected arbitrarily and streaked consecutively at least four times to obtain pure colonies. Strain was maintained on YPD agar plates. The species of PAN was confirmed to be S. cerevisiae by 16S rRNA analysis (Kurtzman 1998).

### 2.2 Cheese whey medium preparation

Cheese whey medium was obtained from the cheese manufacturing by a local dairy factory.
To prevent protein precipitation during heat sterilization, cheese whey, containing a lactose concentration of about 25 g/L and a pH of 3.2, was deproteinized as follow: pH of whey was adjusted to 4.5, boiled at 100 ºC for 15 min and, after cooling, sediment proteins were collected by centrifugation at 5,600 x g at 10 °C for 15 min.
Deproteinized whey was sterilized at 120 ºC for 15 min. Sterile whey was maintained at 4 °C and used as medium in growth experiments, after addition of β-galactosidase (β-gal) (Aspergillus oryzae β-galactosidase, Sigma) at the desired concentration.

### 2.3 Inoculum preparation and growth conditions

Yeast cultures for inoculation were pre-grown in Erlenmeyer flasks filled to 40 % of the total volume with YPD medium. After incubation at 30 °C and 150 rpm for 24 h, the cell suspension was aseptically collected by centrifugation (10 min at 4,000 rpm, 4 °C). Subsequently, yeast cells were resuspended in cheese whey, inoculated into an appropriate volume of cheese whey and incubated at 30 °C and 150 rpm for 24 h.
Growth rates of yeast strains were measured under various conditions by determination of $OD_{600}$ as a function of time using a Tecan Sunrise 96 well Microplate Reader (Tecan, Mannedorf, Switzerland) or a spectrophotometer Genesys TM 20 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA ). Growth curves were carried out in triplicate; data shown are averages of three experiments, standard deviation not exceeded 20 % of the mean.
Growth experiments were conducted also in shake-flasks and in a bioreactor. All experiments were performed in triplicate. Samples of each culture were aseptically removed at regular intervals for chemical analyses. The production of biomass was measured on the basis of cell dry weight (CDW) (g/L) by centrifugation at 4,000 rpm of cell culture and subsequently drying and weighing.
For bioreactor cultivations a BIOSTAT® plus bioreactor system (Sartorius, Göttingen, Germany) equipped with two parallel bioreactors was used with a working volume of 0.7 L. Cultures were carried out in aerobic conditions with air gassing (1 L/min). The stirring speed was 800 ±1 r.p.m., temperature set at 30 ± 0.1 °C and

no pH control active because of the natural buffering capacity of whey. pH and dissolved oxygen values were monitored with two sensors: Easyferm plus K8 (Hamilton, Bonaduz, Switzerland) and OXYFERMTM O2 sensor (Hamilton, Bonaduz, Switzerland), respectively.
For all cultivations, the culture medium was inoculated at a starting OD of 2-3 from an overnight pre-culture.

### 2.4 Analytical procedures

Concentrations of lactose, glucose, galactose and ethanol were determined by HPLC analysis using a Waters Alliance 2695 separation module (Waters, Milford, MA, USA) equipped with a Rezex ROA-Organic Acid H+ (8 %) 300 mm×7.8 mm column (Phenomenex Inc., USA), coupled to a Waters 2410 refractive index detector and a Waters 2996 UV detector. Separation was carried out at 65 °C with 0.005 M $H_2SO_4$ as mobile phase at a flow rate of 0.6 mL/min.

## 3. Results and discussion

A preliminary analysis of *S. cerevisiae* PAN strain, isolated from commercial compressed baker's yeast and identified by 16S rRNA sequence analysis (data not shown), was carried out to determine the extent of its ability to growth on whey.
PAN strain was able to grow on whey with the same growth performance as on YPD (Figure 1) when the hydrolytic enzyme β-gal was added because of the inability of *S. cerevisiae* to use directly lactose.

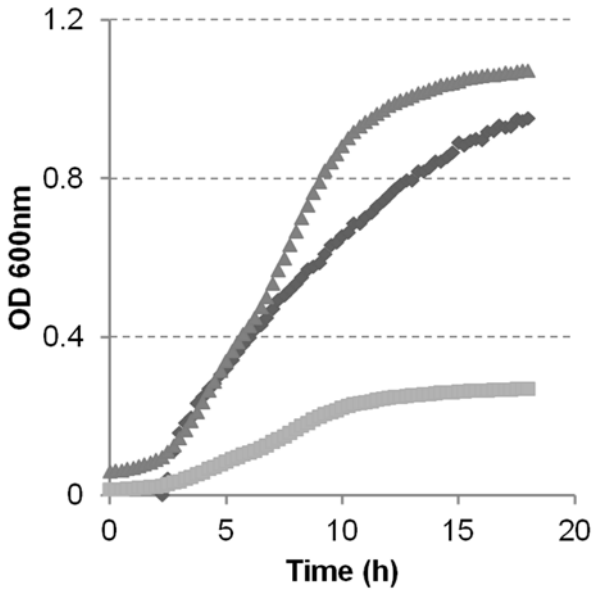


*Figure 1: Growth of PAN strain ($OD_{600}$) as a function of time (h). PAN was grown in YPD liquid culture (♦) or deproteinized whey with (▲) or without (■) β-gal addition, as indicated. Growth curves were performed with microplate method and represent the average of three experiments.*

Cheese whey is an excellent growth medium and contains all the necessary nutrients (including nitrogenous substances, vitamins and trace elements) to sustain cell growth. On the other hand, for *S. cerevisiae* large-scale fermentation, the β-gal employment can be a limiting economical factor. A significant reduction of enzyme concentration may allow a substantial cost reduction in an industrial process. Moreover lower enzyme amount could induce a gradual release and co-consumption of glucose and galactose, increase biomass yield and reduce ethanol production.
To establish the optimal concentration of β-gal for lactose hydrolysis, we carried out microplate growth's experiments in the presence of increasing concentrations of the enzyme. In Figure 2 the specific growth rate (μ) of PAN strain on whey as growth medium is shown.
Compared to the growth on YPD (white bar) we observed an increase of the μ up to 32 UE/mL of β-gal. Higher concentrations lead to a decrease of the specific growth rate. Low concentrations of enzyme (0.25 UE/mL) yielded growth performances comparable to the control (YPD) and a doubling of the specific growth rate was observed only if the β-gal concentration was increased more than 100 times.

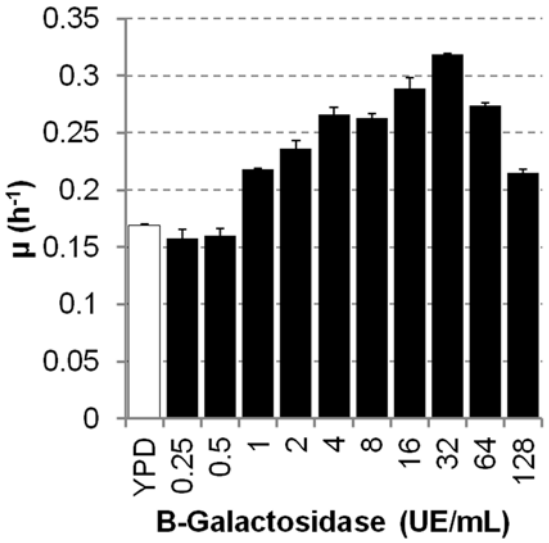


*Figure 2: Specific growth rate (μ) of PAN strain grown on YPD or whey at indicated β-gal concentration. Data represent the means and standard deviations from three independent experiments performed in triplicate.*

The increase of biomass (CDW) and the kinetics of production and consumption of lactose, glucose, galactose and ethanol in the presence of high (2 UE/mL) or low (0.25 UE/mL) enzyme concentrations in shake flask growth experiments (Figure 3 A and B) were measured. High concentrations of β-gal caused the complete and fast hydrolysis of lactose with the release of glucose and galactose which were not co-consumed because galactose metabolism was repressed by the high concentration of glucose achieved (>1 g/L). Only when glucose level failed below the threshold determining the catabolic repression, galactose started to be consumed.

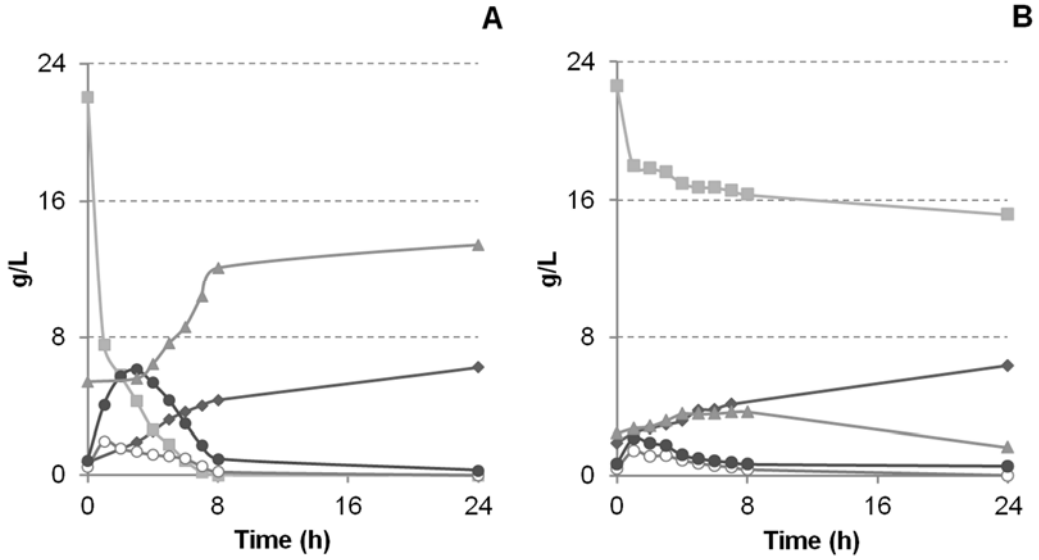


*Figure 3: Shake-flask experiments of cheese whey growth of S. cerevisiae. Cells were grown in deproteinized whey supplemented with 2 UE/mL β-gal (A) or 0.25 UE/mL β-gal (B). Cell dry weight (CDW) (♦), lactose (■), glucose (○), galactose(●) and ethanol (▲).*

Furthermore also a significant ethanol production was observed at high enzymatic concentration; this is due to the Crabtree effect typical of yeast S. cerevisiae (Barford 1979). According to the Crabtree effect, alcoholic fermentation can occur even under strictly aerobic conditions in the presence of excess glucose (as in this case for example as a consequence of the fast release of glucose from lactose). Low concentrations of the

enzyme (0.25 UE/mL) were not sufficient to completely hydrolyse lactose. However, co-fermentation of glucose and galactose was observed. This is a desirable feature from an industrial point of view in terms of increased productivity of process. Ethanol production decreased in agreement with a fine regulation of Crabtree effect in this condition.
The production of biomass in both conditions was comparable even though at low β-gal concentration, the amount of lactose hydrolysed was about 3 times lower with a consequent significant increase of conversion yield.
An increase of the biomass production can be obtained also by controlling the reservoir concentration of glucose and galactose (which led to decreased alcoholic fermentation) or by increasing the oxygen feed (which allows a more active respiration and, consequently, a higher growth yield on glucose and galactose).
In order to control the concentration of the sugars released from lactose hydrolysis and at the same time obtain the complete hydrolysis of lactose, we evaluated the effect of repeated additions of 0.25 UE/mL enzyme on biomass production (Figure 4).

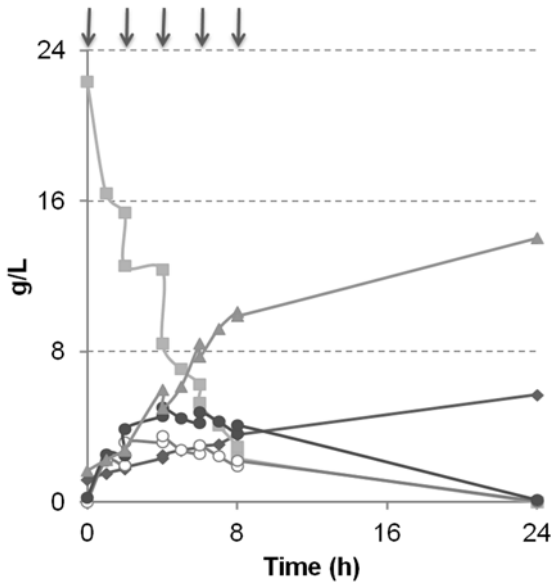


*Figure 4: Shake-flask experiments of cheese whey growth of S. cerevisiae. Cells were grown in deproteinized whey supplemented with 0.25 UE/mL β-gal added every 2 h for the first 8 h (↓). Cell dry weight (CDW) (♦), lactose (■), glucose (○), galactose(●) and ethanol (▲).*

The addition of 0.25 UE/mL, every 2 h for the first 8 h of growth, guaranteed the complete hydrolysis of lactose, furthermore the simultaneous release and consumption of glucose and galactose took place, even though the kinetic of galactose consumption was slightly delayed compared to that of glucose.
It is noteworthy that in these conditions, ethanol production was still observed at the expense of biomass production. This can probably be ascribed to the sub-optimal growth conditions achieved in shake flasks due to the lack of control of aeration and stirring.
In order to evaluate the effects of enzyme limitations using carefully controlled process conditions, we tested the repeated additions of 2 UE/mL β-gal or of 0.25 or 0.125 UE/mL in a bioreactor.
As shown in Figure 5 the rate of lactose hydrolysis was slower as lower the enzyme concentration was. The repeated additions of the enzyme assured the complete hydrolysis of lactose in the first 8 h also at 0.125 UE/mL. At same concentration an optimal control of glucose and galactose release was obtained and a co-consumption of both hexoses was assured.
Ethanol fermentation was minimized in keeping with the fine regulation of Crabtree effect enabled by the repeated additions of enzyme.
The significant increase of biomass production obtained is promising for further development of optimized operative conditions of the fermentation process.

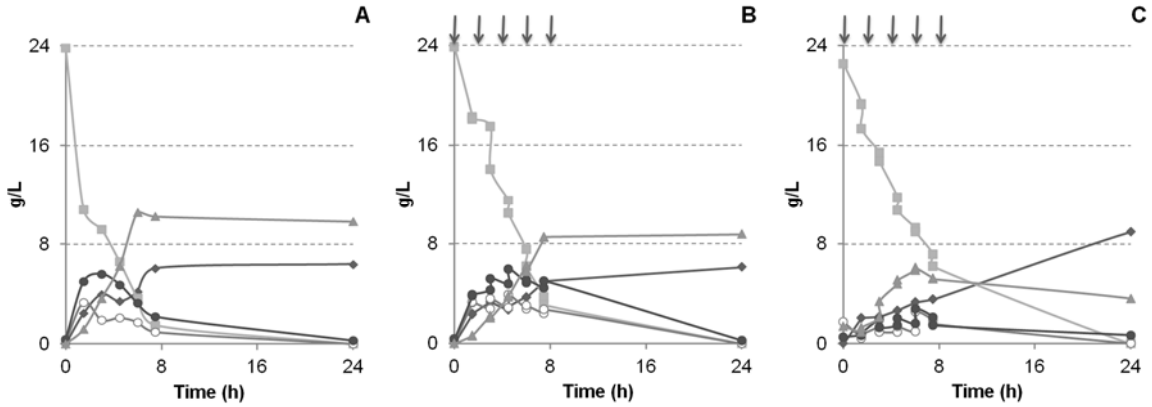


*Figure 5: Bioreactor experiments of cheese whey fermentation by S. cerevisiae. Cells were grown in deproteinized whey supplemented with 2 UE/mL β-gal (A) or 0.25 UE/mL (B) and 0.125 UE/mL (C) β-gal added every 2 h for the first 8 h (↓). Cell dry weight (CDW) (♦), lactose (■), glucose (○), galactose(●) and ethanol (▲).*

## 4. Conclusions

Cheese whey is an excellent medium for growth of yeast S. cerevisiae*,* supplying all required nutrients and carbon source. Hydrolysis of lactose requires the addition of the enzyme β-galactosidase. Since high levels of glucose can inhibit yeast respiration decreasing biomass formation, it is preferable not to convert lactose to glucose and galactose at high rate. Controlled conversion of lactose has many advantages: (i) simultaneous glucose and galactose consumption is faster than that of single sugars; (ii) β-galactosidase repeated additions limit the concentration of glucose and galactose and prevent the onset of Crabtree effect; (iii) the low fermentative activity increases growth yield on glucose and galactose**.** Hence, controlled addition of β-galactosidase has proven to be a promising approach for the production of yeast biomass using cheese whey.

## Acknowledgements

The authors would like to thank CIRCC for financial support. This work was partially supported by the " Reti di Laboratori - Produzione Integrata di Energia da Fonti Rinnovabili nel Sistema Agroindustriale Regionale ” program funded by the Apulia Italian Region.

## References

Barford J.P., Hall R.J., 1979, An Examination of the Crabtree Effect in Saccharomyces cerevisiae: the Role of Respiratory Adaptation, Microbiology, 114 (2), 267-275.

Champagne C.P., Goulet J., Lachance R.A., 1990, Production of bakers' yeast in cheese whey ultrafiltrate, Applied and Environmental Microbiology 56(2), 425-30.

Domingues L., Guimarães P.M., Oliveira C., 2010, Metabolic engineering of Saccharomyces cerevisiae for lactose/whey fermentation, Bioengineered Bugs, 1(3), 164-71.

Ghaly A.E., Rushton D.G., Mahmoud N.S., 2007, Potential air and groundwater pollution from continuous high land application of cheese whey, American Journal of Applied Sciences, 4, 619-627.

González Siso M.I., 1996, The biotechnological utilization of cheese whey: A review, Bioresource Technology, 57 (1), 1-11.

Kurtzman C.P., Robnett C.J., 1998, Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) rDNA partial sequences, Antonie van Leewenhoek, 73, 331-371.

Mollea C., Marmo L., Bosco F., 2013, Valorisation of Cheese Whey, a By-Product from the Dairy Industry, Food Industry, Dr. Innocenzo Muzzalupo (Ed.), InTech, DOI, 10.5772/53159.

Porro D., Martegani E., Ranzi B.M., Alberghina L., 1992, Lactose/whey utilization and ethanol production by transformed Saccharomyces cerevisiae cells, Biotechnolology Bioengineering, 39(8), 799-805.

Sansonetti S., Curcio S., Calabrò V., Iorio G., 2010, Evaluation of the parameters effects on the bioethanol production process from Ricotta cheese whey, Chemical Engineering Transactions 20, 13-18.